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GTTGACTCCGGGGGCGCGGCGAGGAGAGACATGAGGCTGAGCTG
GTTCCGGGTCCTGACAGTACTGTCCATCTGCCTGAGCGCCGTGGC
CACGGCCACGGGGGCCGAGGGCAAAAGGAAGCTGCAGATCGGGG
TCAAGAAGCGGGTGGACCACTGTCCCATCAAATCGCGCAAAGGG
GATGTCCTGCACATGCACTACACGGGGAAGCTGGAAGATGGGAC
AGAGTTTGACAGCAGCCTGCCCCAGAACCAGCCCTTTGTCTTCTC
CCTTGGCACAGGCCAGGTCATCAAGGGCTGGGACCAGGGGCTGC
TGGGGATGTGTGAGGGGGAAAAGCGCAAGCTGGTGATCCCATCC
GAGCTAGGGTATGGAGAGCGGGGAGCTCCCCCAAAGATTCCAGG
CGGTGCAACCCTGGTGTTCGAGGTGGAGCTGCTCAAAATAGAGCG
ACGAACTGAGCTGTAACCAGACTGGGGAGGGGCAGGGGGAGAGG
CCCCCATCAGGGACCAGACTGTTCCAAAAAAAAAACAAAAAACA
AAAACAAACAAAAAAACACTTAAAAGCCAAG

(57) Abstract

A novel prolyl isomerase, RFKBP, which binds FK506 and rapamycin with quantitatively significant selectivity and does not bind cyclosporin A and the m lecular cloning of a cDNA (SEQ ID NO: 6) whose sequence predicts a chemically determined amino acid sequence of RFKBP. The deduced protein of the cDNA is 142 amino acid residues in length (SEQ ID NO: 7).

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RFKBP: A NOVEL PROLYL ISOMERASE AND RAPAMYCIN/FK506 BINDING PROTEIN

Background of the Invention

Cyclosporin A and FK506 are naturally occurring cyclic peptides that inhibit T-cell activation. Although both are potent immunosuppressants, they are structurally unrelated and bind to unique cytosolic proteins: cyclosporin A to cyclophilin and FK506 to FK binding proteins (FKBP's). One recently identified FKBP has an approximate relative molecular mass (M_r) of 11,800 (12K), and a pI of 8.8-8.9 (Harding, M.W. et al., Nature 341:758-760 (1989)). Studies have shown that the unbound 12K FKBP catalyzes the cis-trans isomerization of proline residues in proteins and peptides. However, when 12K FKBP (FKBP12) binds FK506, this activity is inhibited. Whether this inhibition of proline isomerase activity plays a vital role in the immunosuppression of T-cell activation remains to be seen. Recent studies suggest that the FK506-FKBP12 complex (as well as the cyclosporin A-cyclophilin complex) function as immunosuppressants by binding to and inhibiting a protein called calcineurin. (Liu, J.,

et al., Cell 66:807-815 (1991). FK506 is now in human clinical trials and shows remarkable efficacy in solid organ transplantation.

Summary of the Invention

The present invention relates to a structurally novel protein of mammalian origin which binds FK506 and is of low molecular weight. The newly identified FK506 binding protein, referred to herein as rapamycin FK506 binding protein or RFKBP, also binds rapamycin, which is a macrolide structurally related to FK506, but does not bind the immunosuppressant cyclosporin A. RFKBP binds FK506 and rapamycin with quantitatively significant selectivity (i.e., binds FK506 and rapamycin with significantly different quantitative selectivity) and, thus, can be used to differentiate rapamycin-like immunosuppressive agents from FK506-like agents in screening assays or analyses.

As described herein, and in U.S.S.N. 697,113, a novel FK506 binding protein from bovine thymus has been purified and characterized. Also as described herein, a corresponding human cDNA has been cloned from a human placental cDNA library by screening the library with a DNA probe whose sequence was based on the bovine RFKP amino acids sequence.

The novel FK506 binding protein purified from bovine thymus (designated bRFKBP) has been shown to bind FK506 and rapamycin with quantitatively significant selectivity and does not to bind cyclosporin A. The bRFKBP is of low molecular weight (i.e., M_r approximately 16,000, as measured in SDS-PAGE gels), and has been shown to have cis-trans prolyl isomerase activity. In addition, a partial amino acid sequence has been determined for bRFKBP (SEQ ID No: 5)

and amino acid sequence analysis has shown that bRFKBP has greater than 50% homology, in the N-terminal region, to FKBP12.

The human cDNA clone, identified as described herein, has been characterized. In particular, the cDNA insert of the human clone was purified and sequenced in its entirety. The cDNA insert is approximately 562 bp (SEQ ID No: 6). The deduced amino acid sequence of the encoded protein (SEQ ID No: 7) is also presented herein. The encoded protein sequence is 142 amino acid residues and has a calculated M_r of approximately 13,200, which is similar to the M_r of the bovine RFKBP.

Thus, the present invention includes FK506 binding protein of mammalian origin and, particularly FK506 binding protein of non-human (bovine) and human origin. RFKBP of the subject invention binds FK506 and rapamycin with quantitatively significant selectivity. The present invention also relates to DNA and RNA encoding RFKBP, antibodies (polyclonal and monoclonal) which bind RFKBP, and methods of using the foregoing. The present invention also includes RFKBP homologues or equivalents (i.e., proteins which have amino acid sequences substantially similar, but not identical to, that of RFKBP and exhibit FK506, rapamycin and cyclosporin A binding characteristics substantially the same as those described herein for RFKBP). This invention further includes RFKBP peptides (RFKBP fragments which have substantially similar binding characteristics, yet are less than the entire RFKBP amino acid sequence), and uses for the RFKBP homologues, RFKBP peptides, nucleic acid sequences encoding RFKBP homologous or RFKBP peptides and uses thereof.

The RFKBP of the present invention can be used in screening assays to detect new immunosuppressive compounds and, because it binds FK506 and rapamycin with quantitatively significant selectivity, can be used to differentiate rapamycin-like compounds from FK506-like compounds. In addition, RFKBP can be used to identify naturally occurring intracellular substrates which might be targets for other immunosuppressive agents, to identify naturally occurring intracellular rapamycin and FK506-like molecules which function in intrinsic regulatory events in cellular metabolism and to measure parent compounds or metabolites of such compounds in individuals receiving immunosuppressive drug therapy. It can also be used in a counterscreening technique to distinguish potentially toxic compounds from candidate immunosuppressants.

Brief Description of the Drawings

Figure 1 is the partial amino acid sequence of bovine RFKBP (SEQ ID NO: 5) and of four additional FKBPs Neurospora crassa, (SEQ ID NO: 1) human FKBP12, (SEQ. ID NO: 2) bovine FKBP12 (SEQ. ID NO: 3) and Saccharomyces cerevisiae FKBP (SEQ ID NO: 4). Vertical lines indicate identical amino acids at the position indicated and ":" indicates residues observed to form salt bridges in the crystal structure.

Figure 2 is the nucleic acid sequence of the coding strand of full-length cDNA for human placental RFKBP, which is 562 base pairs in length (SEQ ID NO: 6).

Figure 3 is the amino acid sequence of RFKBP, as determined from the ORF sequence. The sequence is 142 residues in length (SEQ ID NO: 7).

Detailed Description

A novel FK506 (RFKBP) binding protein has been purified from bovine thymus (bRFKBP) and shown to be a cis-trans prolyl isomerase. It shares homology with the amino acid sequence of the previously-identified FK506 binding protein of M_r 11,800 (FKBP12), and binds rapamycin, but, unlike FKBP12, binds it with significantly different quantitative selectivity. This novel RFKBP does not bind cyclosporin A.

In addition, a human cDNA which encodes RFKBP has been cloned from a human placental library, its nucleic acid sequence has been determined (SEQ ID NO: 6) and the amino acid sequence of the encoded protein has been deduced (SEQ ID NO: 7). The deduced amino acid sequence of hRFKBP and bRFKBP are homologous, except at the third amino acid (alanine in hRFKBP and threonine in bRFKBP). Therefore it is resonable to expect that hRFKBP also binds FK506 and rapamycin with significantly different quantitative selectivity and does not bind cyclosporin A.

Purification and the Characterization of bRFKBP

As described in Example 1, bovine thymus cytosol extract was passed over an FK506 affinity column containing an amino derivative of FK506 at the C32 position. As a result, several proteins, which were retained by the FK506 matrix and released by FK506 in solution, were obtained. These included proteins of the following approximate molecular weights (M_r): 16,000; 28,000; 32,000; 45,000; 55,000; 60,000; 66,000; and 80,000. Approximate molecular weight was as determined by SDS-PAGE on a 12½% gel using lysozyme (14,400) and α chymotrypsin (21,500) to calibrate relative migration.

N-terminal amino acid sequencing of the protein of approximate molecular weight 16,000 was performed after electrotransfer of the protein to a PVDF membrane, according to the method described by Matsudaira (Matsudaira, P., J. Biol. Chem. 262:10035-10038 (1987)). Additional internal sequence information was obtained by digestion of nitrose cellulose or PVDF membrane-bound protein by endoproteinase Lysine C or cyanogen bromide, followed by microbore HPLC isolation of the resulting peptide fragments. (Matsudaira, P., A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press (San Diego), 1989). The N-terminal and internal amino acid sequences are shown in Figure 1. (SEQ ID NOS: 1-5) Comparison of the amino acid sequence of bRFKBP with the sequence of FKBP12 revealed greater than 50% homology in the N-terminal regions of the two proteins.

Enzymatic properties of homogeneous bRFKBP eluted from the FK506 matrix were also assessed. Homogeneity of bRFKBP was determined by SDS-PAGE and isoelectric focusing. As described in Example 2, the assay of Fisher et al. was used to measure peptidyl prolyl cis-trans isomerization activity of bRFKBP. The activity of FKBP12 and of cyclophilin was also assessed. As shown in Tables 1 and 2 of Example 2, bRFKBP and FKBP12 share a preference for large, hydrophobic residues at the P_1 position (where L>F>V>A). Results shown in Tables 1 and 2 clearly demonstrate that the three isomerases are functionally distinct. Of particular interest are the binding characteristics of RFKBP, as to the binding of rapamycin, FK506 and cyclosporin.

Also as described in Example 2, the ability of rapamycin, FK506 and cyclosporin A to inhibit the isomerase activity of bRFKBP, FKBP12 and cyclophilin

was assessed, using standard techniques. As shown in Table 3 and 4, RFKBP has a K_i of 180 nM and 38 nM, respectively, for FK506 and a K_i of 6.3 nM and 3.6 nM, respectively, for rapamycin. These results indicate that rapamycin is a more potent inhibitor of isomerase activity than is FK506, and that bRFKBP does not bind cyclosporin A at all. bRFKBP and FKBP12 were not inhibited by cyclosporin A, and cyclophilin was not inhibited by rapamycin.

Cloning of cDNA Encoding hRFKBP

To facilitate the isolation of a human cDNA clone containing a DNA insert which hybridizes to DNA encoding bRFKBP, DNA probes were designed as described in Example 3. Two degenerate oligonucleotides (SEQ ID NOS: 8 and 9) were designed based on the determined protein sequence of bRFKBP. These DNA oligomers were then used as polymerase chain reaction primers to amplify the DNA fragment. The amplified fragment was then cloned into a cloning vector and its DNA sequence determined. This DNA fragment was then excised from the vector, radiolabeled with ^{32}P and used to screen a human placental cDNA library (Stratagene, Catalog #936203).

As described in Example 3, a human cDNA clone containing an approximately 600 base pair insert which hybridizes with a DNA fragment encoding an amino acid sequence present in bRFKBP has been identified, purified, and sequenced. This sequence is presented in Figure 2 (SEQ ID NO: 6).

The correct open reading frame of the cDNA sequence encoding hRFKBP was identified (see Example 3). The deduced amino acid sequence, from amino terminus to carboxyl terminus, is shown in Figure 3

(SEQ ID NO: 7). The deduced protein has 142 amino acids.

The deduced amino acid sequence of human placental RFKBP and the chemically determined sequence of bovine thymus RFKBP are homologous, except at the third amino acid (alanine in hRFKBP and threonine in bRFKBP).

Enzymatic properties of the hRFKBP protein encoded by the isolated cDNA can also be assessed using the methods as described in Example 2. For example, the assay of Harrison and Stein (Harrison, R.K. and R.L. Stein, Biochemistry 29:3813-3816 (1990)) can be used to measure peptidyl prolyl cis-trans isomerization activity of hRFKBP as was done for bRFKBP. Also as described in Example 2, the ability of FK506, rapamycin and cyclosporin A to inhibit isomerase activity of RFKBP can be assessed using standard techniques. The results of these two assays are clearly useful to demonstrate specific FK506 isomerase activity which is selectively inhibited by the presence of FK506 and rapamycin.

Thus, as a result of the work described herein, a new FK506 binding protein of mammalian origin, which is of different size and binding specificity than the previously described FKBP12, is available. It is a new member of the class of prolyl cis-trans isomerases which are key regulators of immune responses and, thus, has a variety of uses. As shown in Figure 1, the bovine RFKBP shares significant sequence homology with the sequences of four known FKBPs, yet this bRFKP is selectively inhibited by rapamycin and FK506.

In addition, a human cDNA clone containing a cDNA insert which hybridizes with a DNA fragment encoding a bRFKBP amino acid sequence has been obtained and its deduced amino acid sequence has been shown to be

essentially identical to the amino acid sequence of bRFKBP.

The hRFKBP protein encoded by the cDNA insert is shown in Figure 2 (SEQ ID NO: 6). The amino acid sequences of the human and bovine RFKBP are essentially identical, with only a single amino acid distinguishing the two. It is reasonable to believe, given the essentially identical sequences of bRFKBP and hRFKBP, that hRFKBP shares the same selective binding and inhibitor properties of bRFKBP. The RFKBP proteins of this invention include analogous or homologous amino acid sequences which encode proteins having the capability of selectively binding FK506 and rapamycin. These proteins also include those which exhibit substantially the same binding capabilities as bRFKBP or hRFKBP and in which the amino acid sequence differs from that of bRFKBP or hRFKBP by a deletion, addition or substitution. These proteins further include sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration.

Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar amino acids include serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The charged (basic) amino acids include arginine, lysine, and

histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

In addition, the protein structure can be modified by deletions, additions, inversion, insertions or substitutions of one or more amino acid residues in the sequence which do not substantially detract from the desired functional properties of the peptide. Naturally occurring allelic variations and modifications are included within the scope of the invention so long as the variation does not substantially reduce the ability of the protein to selectively bind FK506 or rapamycin.

Modified RFKBP proteins capable of selectively binding FK506 or rapamycin can be made using recombinant DNA techniques, such as expression of the protein from a vector containing cDNA encoding such as protein, or by synthesizing DNA encoding the desired protein mechanically and/or chemically using known techniques.

An alternate approach to producing the RFKBP proteins of this invention is to use peptide synthesis to make a peptide or polypeptide having the amino acid sequence of such a protein. The peptides or modified equivalents thereof can be synthesized directly by standard solid or liquid phase chemistries for peptide synthesis. For example, the above amino acid sequence or modified equivalent thereof encoding the RFKBP protein can be synthesized by the solid phase procedure of Merrifield.

Preferably, the RFKBP protein will be produced by inserting DNA encoding the protein into an appropriate vector/host system where it is expressed. The DNA sequences can be chemically synthesized or they can be obtained from natural sources using recombinant technology.

A variety of host-vector systems can be used to express the protein of this invention. Host-vector systems include, but are not limited to, the following: bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA.

Any of the standard recombinant methods for the insertion of DNA into an expression vector can be used. The recombinant DNA vector can be introduced into an appropriate host cell by any method appropriate for that host cell, such as transformation, transduction, transfection, or electroporation and the resulting host cultured to express the proteins of this invention.

This invention also pertains to an expression vector comprising a DNA sequence encoding for a protein which exhibits RFKBP-like activity having the capability of binding FK506 and rapamycin and cells transformed thereby.

The RFKBP protein expressed by the cDNA clone can be used for a number of diagnostic and therapeutic purposes. The RFKBP can be used in screening assays for detection of new naturally occurring immunosuppressant compounds. For example, RFKBP could be used to screen fermentation broths, produced by known techniques, for compounds that bind to it and thus are potential immunosuppressant candidates. Alternately, RFKBP can be used to screen existing synthetic compounds for binding affinity and subsequent immunosuppressant evaluation. That is, by using RFKBP in a screening assay, rapamycin-like immunosuppressive agents can be distinguished from FK506-like immunosuppressive agents because of the binding differential, as represented in the Tables of Example 2. This differential screening is not possible using the previously identified FKBP12 because it binds FK506 and rapamycin with similar affinity, as represented in

Tables 3 and 4. When used in a screening method, RFKBP will generally be bound to an appropriate solid support, as described below with reference to diagnostic uses. It is reasonable to expect that a compound which binds to RFKBP will be FK506-like and, thus, have immunosuppressive capabilities.

RFKBP can also be used as the basis for design of FK506-like molecules by determining and characterizing the active binding site(s) of RFKBP and designing a molecule which binds to it (them) and assessing its (their) ability to suppress an immune response.

It is also possible to use the newly identified RFKBP for diagnostic purposes. For example, RFKBP can be affixed to a solid support using a variety of chemical coupling techniques which link amino acid residues, such as methionine, lysine, cystine, and tryptophan to inert matrixes, such as Affigel (BioRad) or cyanogen bromide-treated Sepharose (Pharmacia). The RFKBP bearing solid support is then contacted with tissue extracts or body fluids, such as blood and urine, from individuals receiving FK506 immunosuppressant treatment. Detection and/or quantitation of the parent compound FK506, or its metabolites, can be carried out using known methods, such as spectrophotometric measurement or scintillation counting.

It is also possible to use RFKBP to identify natural intracellular FK506-like molecules (as well as other molecules exhibiting immunosuppressant-like activity) that function in intrinsic regulatory events in cellular immunity and metabolism. Furthermore, RFKBP can be used to identify natural intracellular substances that may be targets for other novel immunosuppressive agents.

Finally, it may be possible to modify RFKBP in such a way as to enhance its binding capability, and/or other immunosuppressive characteristics. Such modifications (e.g., truncating sequence length) can be carried out using known methods, such as site directed mutagenesis.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

Example 1 Purification of bRFKBP

An amino derivative of FK506 at the C32 position was prepared as described in Fretz et al. (Fretz, H., et al., J. Am. Chem. Soc. 113:1409-1411 (1991)) and coupled to Affigel 10 resin to yield a FK506 affinity matrix (approximately 1 mg of FK506 coupled per ml of resin). Bovine thymus cytosol extract was prepared as follows: tissues were snap frozen in liquid nitrogen and 75 gram amounts were homogenized in 100 mM potassium phosphate containing 1 mM PMSF and 5 mM DTT for 60 sec in a Waring blender. The extract was clarified by centrifugations at 20,000 rpm and then 100,000 x g. Cytosol extract was then passed over a 5 ml FK506 affinity column at 0.2 ml/min. The column was washed extensively with phosphate buffered saline containing 0.1% Tween 80 detergent and eluted sequentially with FK506 (200 ug/ml in phosphate buffer) and then 6 M guanidine hydrochloride. Eluted proteins were dialyzed extensively against 10 mM Tris, pH 7.0 and aliquots were lyophilized. SDS-PAGE analysis revealed that several proteins were retained by the FK506 matrix and released by FK506 in solution.

Example 2 Assessment of Enzymatic Properties of bRFKBP

To assess enzymatic activity, proteins eluted from the FK506 matrix were resolved by reverse phase HPLC on a C4 column (Vydac). Homogeneity of RFKBP was determined by SDS-PAGE and N-terminal amino acid sequencing. Prolyl cis-trans isomerase activity was measured using the assay described by Fisher et al. and succinyl-Ala-Leu-Pro-Phe-p-nitroaniline as substrate (Fisher, B. et al. Nature 337:476-478 (1989)). A typical kinetic experiment was performed in a final volume of 1.0 ml 100 mM Tris, pH 7.8 at 15°C. Enzymes and substrate were present at the following concentrations: Isomerase- 5-20 nM; chymotrypsin- 100 ug/ml; peptidyl substrate- 30 uM. Reaction progress was monitored by the absorbance change at 405 nM for 5 min using an HP Model 8452A Diode Array Spectrophotometer. First order rate constants were determined and K_i values calculated with the FITKIN (P. Kuzmic, University of Wisconsin) data analysis program. Results are presented in Tables 1-4. Tables 1 and 2 show the specific activity of the peptidy prolyl cis-trans isomerase activity of bRFKBP for different peptide substrates. Tables 3 and 4 indicate cis-trans prolyl isomerase activity and K_i's of 180 and 38 nM respectively and 6.3 and 3.6 nM respectively for FK506 and rapamycin. These K_i's indicate a significant difference in binding preference between FK506 and rapamycin. For contrast, FKBP12 has K_i's of 0.4 and 0.5 nM and 0.2 and 0.25 for FK506 and rapamycin, respectively. These same assays may be used to assess specificity of the hRFKBP.

TABLE 1

Comparison of bRFKBP, FKBP-12 and Cyclophilin Peptidyl Prolyl Cis-trans Isomerases for Peptide Substrates of the Sequence Suc-Ala-Xaa-Pro-Phe-p-nitroanaline

	RFKBP1		FKBP-12[1]	Cyclophilin[2]
Substrate	Batch 1	Batch 2		
ALPF	7.2*	2.8	2.6	2.7
ALPF		0.66	0.620	1.39
AKPF		0.44	0.028	0.92
AVPF		0.17	0.170	3.18
AAPF		0.05	0.053	3.18

*$K_{cat}/K_m \times 10^6$ ($M^{-1}s^{-1}$)

[1] Determined at 15°C

[2] Determined at 10°C

TABLE 2

Specificity of the Peptidyl Prolyl Cis-Trans Isomerase Activity of FKBP-12 and bRFKBP for Suc-Ala-Xaa-Pro-Phe-p-NA Substrates

(k_c/K_m in $M^{-1}s^{-1}$ at 15°C)

Xaa	FKBP-12	bRFKPB
Leu	4.33×10^6	2.08×10^6
Phe	2.17×10^6	6.65×10^5
Val	9.17×10^5	9.24×10^4
Ala	3.17×10^5	1.10×10^5
Lys	1.60×10^5	4.20×10^5
Gly	3.50×10^3	9.28×10^3 (estimate)
Glu	1.55×10^3	1.56×10^4 (estimate)

TABLE 3

Comparison of Ic_{50} Values for Inhibition of cis-trans Prolyl Isomerase Activity of bRFKBP, FKBP-12 and Cyclophilin by Rapamycin, FK506 and Cyclosporin A

Inhibitor	RFKBP	FKBP-12	Cyclophilin
Rapamycin	6.3 nM	0.2 nM	NI*
FK506	180 nM	0.4 nM	NI
Cyclosporin A	NI	NI	2.0 nM

* NI = not inhibited at drug concentrations of 420 nM.

TABLE 4

Inhibition of the Peptidyl Prolyl Cis-Trans Isomerase Activity of FKBP-12 and bRFKBP by FK506 and Rapamycin

(K_i in nM at 15°C)

Inhibitor	FKBP-12	bRFKBP	Ratio bRFKBP/FKBP
FK506	0.5	38	80
Rapamycin	0.25	3.6	14

Example 3 cDNA Cloning of hRFKBP

Two degenerate oligonucleotides, (SEQ ID NOS: 8 and 9, respectively) were designed based on the previously determined bRFKBP sequence and were synthesized on an Applied Biosystems 380A DNA Synthesizer. Using the degenerate oligonucleotides as primers for the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), a fragment of approximately 270 base pairs (bp) was amplified from a λgt11 human thymus cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) after lysing the phage at 80°C for 20 minutes. The fragment was subcloned into the TA Cloning pCR vector (Invitrogen, San Diego, CA), and eleven potential recombinant colonies were picked and grown overnight on LB plates that contained kanamycin (50mg/ml) and were spread with x-gal (30 ml of 50 mg/ml). Each colony was used to inoculate 5 ml of Luria Broth (LB) containing kanamycin, and the cultures were grown overnight at 37°C.

The DNA from each culture was purified using a Qiagen tip-20 column according to the manufacturer's instructions (Qiagen, Inc., Chatsworth, CA), and the nucleotide sequences of the individual inserts were determined by the dideoxy method (Sanger et al., 1977) using the Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH) and three oligonucleotide primers that hybridized near the vector cloning site. Two of the primers, M13 "reverse" an M13 "forward", were available commercially; the third primer, (SEQ ID NO: 10), was synthesized based on vector sequence so that it would hybridize farther from the cloning site than the M13 forward primer.

Three clones contained DNA inserts with nucleotide sequences that corresponded to the known peptide sequences. DNA from one recombinant clone was digested

with EcoRI and Hindlll endonucleases (New England BioLabs, Beverly, MA), and the insert was purified on a horizontal gel of 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) low melting temperature agarose. The purified DNA fragment was radiolabeled with ^{32}P via a commercial oligolabelling kit (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and was used to probe the λgt11 human thymus cDNA library under stringent conditions (Sambrook et al., 1989). One positive clone was selected and repurified by two additional screens.

The approximate 600 bp insert was amplified by PCR using λgt11 forward and reverse primers (New England Biolabs) and was subcloned into the TA Cloning pCR vector for sequencing. The nucleotide sequence revealed no stop codon in the known open reading frame, indicating that the cDNA was incomplete at the 3′ end. The cDNA insert was radiolabeled with ^{32}P and used to probe a human placental cDNA λZapII library (Stratagene, La Jolla, CA) under stringent conditions. Eight of twenty-two original positives rescreened, and the inserts from two clones were excised according to the manufacturer's protocol. Complete DNA sequences were determined for the cloned inserts using the Sequenase Version 2.0 kit. Each insert predicted an approximate 16 kDa protein that contained the chemically-determined peptide sequence.

Example 4 DNA Sequence Determination of hRFKBP

A full length cDNA encoding hRFKBP (Figure 2) was isolated from a human placenta library as described in Example 3. The 562 bp sequence (SEQ ID NO: 6) of this cDNA contains an open reading frame of 426 nucleotides that begins at the ATG initiation codon and terminates at the TAA codon. The open reading frame is flanked by 30 bp of 5′ untranslated sequence and 106 bp of 3′ untranslated sequence.

Example 5 Deduced Amino Acid Sequence of hRFKBP

The deduced amino acid sequence of human placental RFKBP encodes a 142 residue protein (SEQ ID NO: 7) with a predicted molecular weight of 15,700. The first 22 N-terminal residues are not present in the naturally isolated bovine thymus protein. This presumed leader, or signal, sequence is cleaved to yield a mature protein of 120 residues with a predicted molecular weight of 13,200. The deduced amino acid sequence of hRFKBP and the chemically determined sequence of bovine thymus RFKBP are homologous, except that the third amino acid is alanine in hRFKBP and threonine in bRFKBP.

CLAIMS

1. Homogeneous protein of mammalian origin which binds FK506 and rapamycin with quantitatively significant selectivity and does not bind cyclosporin A.

2. The homogeneous protein of Claim 1 which is of bovine origin and of approximate M_r 16,000.

3. The homogeneous protein of Claim 2 which is encoded in part by the amino acid sequence of SEQ ID NO: 5.

4. The homogeneous protein of Claim 2 which has a K_i of approximately 38 nM for FK506 and a K_i of approximately 3.6 nM for rapamycin.

5. The homogeneous protein of Claim 1 which is of human origin and approximate M_r 13,200.

6. A cDNA clone of mammalian origin in which the cDNA insert hybridizes to a DNA fragment whose sequence encodes a protein which binds FK506 and rapamycin with quantitatively significant selectivity and does not bind cyclosporin A.

7. A cDNA clone of mammalian origin in which the cDNA insert hybridizes to a DNA fragment whose sequence (SEQ ID NO: 6) encodes an amino acid sequence present in RFKBP.

8. A cDNA clone of Claim 7 in which the cDNA insert encodes the amino acid sequence SEQ ID NO: 7.

9. Isolated DNA which hybridizes to DNA which encodes a protein of mammalian origin which binds FK506 and rapamycin with quantitatively significant selectivity and does not bind cyclosporin A.

10. An isolated DNA sequence of mammalian origin encoding a protein that binds FK506 or rapamycin with quantitatively significant selectivity and does not bind cyclosporin A.

11. An isolated DNA sequence of Claim 10 of bovine origin.

12. An isolated DNA sequence of Claim 10 of human origin.

13. An isolated DNA sequence of Claim 12 comprising the nucleotide sequence of SEQ ID NO: 6.

14. A DNA expression vector containing a DNA sequence (SEQ ID NO: 6) encoding a protein of human origin that binds FK506 or rapamycin with quantitatively significant selectivity.

15. A cell transformed with the expression vector of Claim 14.

16. A method of identifying an FK506-like substance, comprising the steps of:

a) combining a substance to be tested and RFKBP which is bound to a solid support;

b) maintaining the product of (a) under conditions appropriate for binding of RFKBP and substances which bind RFKBP; and

c) determining whether binding of RFKBP and a substance which binds RFKBP occurred in step (b), wherein binding is indicative of the presence of an FK506-like substance.

17. The method of Claim 16 wherein RFKBP is bound to the solid support by a chemical linkage to an amino acid residue.

18. The method of Claim 16 wherein the FK506-like substance is not a rapamycin-like substance.

19. A method of detecting FK506 and FK506-like substances in a biological sample, comprising the steps of:

a) combining the biological sample with RFKBP which is bound to a solid support;

b) maintaining the product of (a) under conditions appropriate for binding of RFKBP and a substance which binds RFKBP; and

c) determining whether binding of RFKBP and a substance which binds RFKBP occurred in step (b), wherein binding is indicative of the presence of FK506.

Figure 1

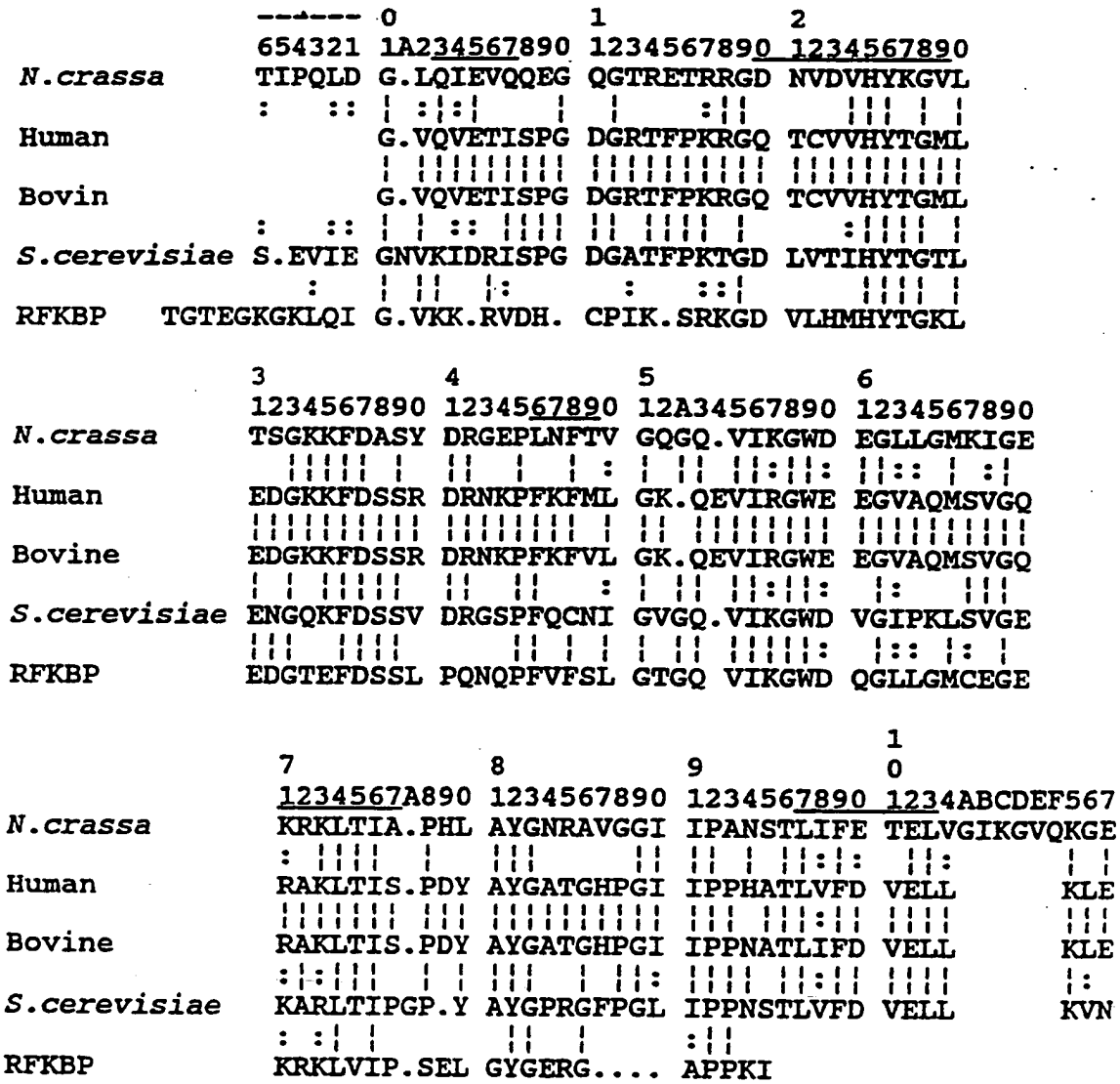
654321 1A234567890 1234567890 1234567890
N.crassa TIPQLD G.LQIEVQQEG QGTRETRRGD NVDVHYKGVL
Human G.VQVETISPG DGRTFPKRGQ TCVVHYTGML
Bovin G.VQVETISPG DGRTFPKRGQ TCVVHYTGML
S.cerevisiae S.EVIE GNVKIDRISPG DGATFPKTGD LVTIHYTGTL
RFKBP TGTEGKGKLQI G.VKK.RVDH. CPIK.SRKGD VLHMHYTGKL
1234567890 1234567890 12A34567890 1234567890
N.crassa TSGKKFDASY DRGEPLNFTV GQGQ.VIKGWD EGLLGMKIGE
Human EDGKKFDSSR DRNKPFKFML GK.QEVIRGWE EGVAQMSVGQ
Bovine EDGKKFDSSR DRNKPFKFVL GK.QEVIRGWE EGVAQMSVGQ
S.cerevisiae ENGQKFDSSV DRGSPFQCNI GVGQ.VIKGWD VGIPKLSVGE
RFKBP EDGTEFDSSL PQNQPFVFSL GTGQ VIKGWD QGLLGMCEGE
1234567A890 1234567890 1234567890 1234ABCDEF567
N.crassa KRKLTIA.PHL AYGNRAVGGI IPANSTLIFE TELVGIKGVQKGE
Human RAKLTIS.PDY AYGATGHPGI IPPHATLVFD VELL KLE
Bovine RAKLTIS.PDY AYGATGHPGI IPPNATLIFD VELL KLE
S.cerevisiae KARLTIPGP.Y AYGPRGFPGL IPPNSTLVFD VELL KVN
RFKBP KRKLVIP.SEL GYGERG.... APPKI

Figure 2

GTTGACTCCGGGGGCGGCGAGGAGACATGAGGCTGAGCTG
GTTCCGGGTCCTGACAGTACTGTCCATCTGCCTGAGCGCCGTGGC
CACGGCCACGGGGGCCGAGGGCAAAAGGAAGCTGCAGATCGGGG
TCAAGAAGCGGGTGGACCACACTGTCCCATCAAATCGCGCAAAGGG
GATGTCCTGCACATGCACTACACGGGGAAGCTGGAAGATGGGAC
AGAGTTTGACAGCAGCCTGCCCCAGAACCAGCCCTTTGTCTTCTC
CCTTGGCACAGGCCAGGTCATCAAGGGCTGGGACCAGGGGCTGC
TGGGGATGTGTGAGGGGGAAAAGCGCAAGCTGGTGATCCCATCC
GAGCTAGGGTATGGAGAGCGGGGAGCTCCCCCAAAGATTCCAGG
CGGTGCAACCCTGGTGTTCGAGGTGGAGCTGCTCAAAATAGAGCG
ACGAACTGAGCTGTAACCAGACTGGGGAGGGGGCAGGGGGAGAGG
CCCCCATCAGGGACCAGACTGTTCCAAAAAAAAAAACAAAAAACA
AAAACAAACAAAAAAACACTTAAAAGCCAAG

MRLSWFRVLTVLSICLSAVATATGAEGKRKLQIGVKKRVDECPIKSRKG
DVLHMHYTGKLEDGTEFDSSLPQNQPPFVFSLGTGQVIKGWDQGLLG
MCEGEKRKLVIPSELGYGERGAPPKIPGGATLVFEVELLKIERRTEL

Figure 3

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/61; C12N9/90; C12N5/10; C12Q1/533

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY. vol. 113, 13 February 1991, GASTON, PA US pages 1409 - 1411; H.FRETZ ET AL: 'Rapamycin and FK506 binding proteins (Immunophilins)' see the whole document	1-19
A	WO,A,9 104 321 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 4 April 1991	6-15
A	EP,A,0 379 342 (MERCK & CO) 25 July 1990 see page 3, line 11 - line 15 see page 11, line 48 - line 50; claim 10	16-19
A	WO,A,8 912 644 (ASSOCIATES OF CAPE COD) 28 December 1989 see page 29-30; claims 21-25	16-19

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 SEPTEMBER 1992	25.09.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN DER SCHAAL C.A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	TRANSPLANTATION PROCEEDINGS vol. 23, no. 6, December 1991, pages 2890 - 2893; S.L. ROSBOROUGH ET AL: 'Identification of FKBP-related proteins with antibodies of predetrmined specificity and isolation by FK 506 affinity chromatography' see page 2892	1-19
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, August 1991, WASHINGTON US pages 6677 - 6681; Y-J JIN ET AL: 'Molecular cloning of a membrane-associated human FK506- and rapamycin-binding protein, FKBP-13' see the whole document	1-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203993 SA 60700

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 10/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9104321	04-04-91	None		
EP-A-0379342	25-07-90	CA-A-	2008149	19-07-90
		JP-A-	2273181	07-11-90
		US-A-	5109112	28-04-92
WO-A-8912644	28-12-89	AU-A-	3772289	12-01-90
		EP-A-	0379549	01-08-90
		JP-T-	3501733	18-04-91